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Seletracetam enhances short term depression *in vitro*

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ABSTRACT

Seletracetam (SEL), an analog of the antiepileptic drug levetiracetam (LEV), decreases seizure activity in a number of epilepsy models and binds to the synaptic vesicle protein SV2A with a higher affinity than LEV. Experiments were performed to determine if SEL, like LEV, reduces the later EPSPs in long trains of stimuli in a manner dependent upon access to the interior of synaptic vesicles and SV2A binding. When hippocampal slices were incubated in 3–30 μM SEL for 3 h, but not 30 min, the relative amplitude of the CA1 field excitatory synaptic potentials decreased over the course of a train of high frequency stimuli more than for control slices. This short term depression was frequency and dose dependent and largely disappeared when the spontaneous activity during the loading period was removed by cutting the Schaffer collaterals. The SEL effect was also observed in slices loaded during prolonged stimulation at 1 Hz, but not 10 Hz. Hippocampal slices loaded with both SEL and FM1-43 to visualize synaptic boutons released the FM1-43 in response to prolonged stimulation much more slowly than control slices during prolonged stimulation. Like LEV, SEL produced a frequency-dependent decrement of synaptic transmission that was dependent upon the drug entering recycling synaptic vesicles and compatible with SV2A binding. Previous observations of SV2A binding affinity correlated with the current effect of SEL and the previously reported effect of LEV on synaptic transmission validate SV2A as an extremely attractive target for future antiepileptic drug development.

1. Introduction

Seletracetam (SEL) along with brivaracetam and levetiracetam (LEV, Keppra™) belongs to a class of compounds that have antiepileptic properties. LEV is frequently prescribed for seizure prevention in patients with partial onset and generalized epilepsy (Kaminski et al., 2009; Wright et al., 2013; Dionisio et al., 2013; Glauser et al., 2013). Despite the broad use of LEV, its detailed mechanism(s) of action remains unknown. All of these compounds bind to SV2A, a synaptic vesicle associated protein, but SEL and brivaracetam bind at least one log unit more strongly than LEV (Lynch et al., 2004; Frycia et al., 2010; Gillard et al., 2011). SV2A binds to synaptotagmin, multiple adapter proteins, CAMKII, an endophilin-like protein and an EGF receptor (Yao and Bajjalieh, 2008; Yao et al., 2010), leading to many possible downstream consequences for vesicle release if its function becomes compromised. Manipulating SV2A expression has not yet provided a fully consistent explanation for the role of SV2A in synaptic vesicle release, nor indicated how the interactions of these compounds with SV2A depresses seizure activity (Janz et al., 1999; Custer et al., 2006; Wan et al., 2010; Nowack et al., 2011).

As a structurally similar compound, SEL may be mechanistically similar to LEV. Both compounds decreased seizure activity in kindled, audiogenic and genetic absence models of epilepsy, but not in maximal electroshock or pentylenetetrazol models (Klitgaard et al., 1998, 2003; Matagne et al., 2009). Like LEV, SEL does not interact directly with postsynaptic glutamate or GABA receptors but potently inhibits negative modulation of glycine currents (Rigo et al., 2002, 2005). Both compounds inhibit calcium currents, but not sodium currents, with SEL doing so at lower concentrations (Zona et al., 2001; Martella et al., 2009; Vogl et al., 2012). Unlike LEV, SEL does not inhibit negative modulation of GABA currents (Rigo et al., 2002, 2005).

None of these actions, however, involve a role for these compounds binding to SV2A. Binding to SV2A is a more likely

Abbreviations: ACSF, artificial cerebrospinal fluid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; fEPSP, field excitatory postsynaptic potential; LEV, levetiracetam; SEL, seletracetam; STD, short term depression; SV2A, synaptic vesicle protein 2A.

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explanation for LEV decreasing synaptic responses to repetitive high frequency stimulation as demonstrated in recordings from hippocampal slices (Yang et al., 2007; Yang and Rothman, 2009; Meehan et al., 2011, 2012). This behavior appears to depend upon LEV gaining access to SV2A *via* uptake into synaptic vesicles during endocytosis (Meehan et al., 2011, 2012). This highly unusual route of entry into neurons has only been reported for exotoxic agents such as botulinum toxin (Bercsenyi et al., 2013). The experiments described below were done to determine whether SEL has similar effects on synaptic transmission as LEV, *but at lower concentrations*, consistent with both compounds exerting their primary action through SV2A. If both compounds work comparably *via* an SV2A-related mechanism, this would strongly reinforce SV2A as an important site for antiepileptic drug discovery.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma (St. Louis, MO) except for 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris, Ellisville, MO), FM 1-43 (Invitrogen, Eugene, OR), ADVASEP 7 (CyDex Pharmaceuticals, Lenexa, KS) and SEL which was supplied by UCB SA, Belgium.

2.2. Animals and slice preparation

Care and use of animals conformed to national and international guidelines as approved by the University of Minnesota Institutional Animal Care and Use Committee. Brains were rapidly and appropriately harvested from 4 to 6 week old Sprague-Dawley rats and briefly immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, and 10 glucose, and continuously bubbled with a 95% O_2/5% CO_2 gas mixture. Transverse 500 μm slices through cortex and hippocampus were cut as previously reported (Yang and Rothman, 2009). In brief, the hemispheres of each slice were separated and incubated in a submerged, oxygenated holding chamber at 25 °C for 0.5 or 3 h before transfer to a submerged recording chamber for electrophysiology or imaging. The holding chambers were filled with ACSF or ACSF containing 1, 3, 10, or 30 μM SEL.

2.3. Electrophysiology

Electrophysiological recordings were made in a submerged chamber perfused at 2 ml min^{-1} with oxygenated, control ACSF or ACSF containing SEL, heated to 26–28 °C (SH-27B, Warner). Extracellular field potentials were recorded in the area of the stratum radiatum of CA1 with glass electrodes (4–6 Mohms) filled with ACSF connected to a DC amplifier (Axoclamp 2A; Axon Instruments, Union City, CA, USA). Bipolar tungsten stimulating electrodes were placed in the area of the Schaffer collateral pathway.

At the start of experiments, an input–output curve was established for the amplitude of the field EPSP (fEPSP), from which half-maximal stimulation intensity was determined. This stimulation strength was used for the remainder of the recording session. Field potentials from the dendritic layer of CA1 were digitized at 1 kHz, and stored on a personal computer using a commercially available A/D converter and software (Digidata 1200 and pClamp 9; Axon Instruments). The magnitude of individual field potentials was measured between onset and peak negative deflection (Fig. 1). Absolute fEPSP amplitudes vary from slice to slice depending upon electrode placement and stimulation intensity and cannot be used to reliably report the effects of SEL. To compare across slices, all fEPSP amplitudes were normalized to the initial fEPSP amplitude in a train. Individual stimuli of 100 μs duration were delivered in trains of 20 pulses at 5, 10, 20, or 40 Hz (0.2, 0.1, 0.05 or 0.025 s interpulse intervals; 4, 2, 1 or 0.5 s total duration/train, respectively). Three trains of 20 stimuli at the same frequency separated by 6 min were averaged and the ratio of the "*x*"th to the first postsynaptic potential ($fEPSP_x/fEPSP_1$) were calculated. Normalizing the field potentials permitted comparison of responses across different slices and experimental conditions. The number of repetitive stimulations was limited to avoid inducing long term potentiation. The amplitudes of fEPSPs were determined offline using custom-programmed software in Matlab (The MathWorks, Natick, MA) as previously described (Meehan et al., 2011).

2.4. Fluorescence microscopy

In these experiments, hippocampal slices that had been incubated in either control ACSF or ACSF containing SEL were transferred to the recording chamber and positioned on the stage of a two-photon microscope (Prairie Technologies, Middleton WI). Perfusion and electrode placements were as above. As previously reported (Yang et al., 2005), the recording electrode was filled with ACSF plus 10 μM of the membrane-selective fluorescent styryl dye FM1-43. The FM1-43 dye was injected pneumatically into CA1 for 60 s before and 30 s after a 10-Hz, 2-min loading stimulus delivered by the bipolar electrode at half-maximum intensity. The slice was then washed for 25 min with ACSF plus 100 μM ADVASEP 7 to facilitate removal of nonspecific dye staining (Kay et al., 1999). From this point, the ACSF contained ADVASEP 7 plus 10 μM CNQX to prevent spontaneous activity and dye release. The fluorescent axonal terminals were visualized using an Olympus 40× water immersion objective (LUMPlan FL/IR, 0.80NA; Tokyo, Japan) and a two-photon Chameleon laser (Coherent, Santa Clara, CA). Stacks of five Images 1 μm apart in the *z*-plane were collected at each time point to allow for a range of imaging levels in the event that the slice shifted in the *z*-direction during the imaging period. Images (512 × 512 pixels, 0.465 μm/pixel) were collected every 1 min for 15 min during a continuous 1 Hz unloading stimulus. To completely unload all remaining dye inside vesicles, an additional 4 min 10 Hz stimulus followed by a 10 min wash occurred before a final "background" stack of images was collected. Images were analyzed using MetaMorph V7.5 (Molecular Devices, Sunnyvale, CA). The fluorescence intensity of FM1-43 within 100 × 100 pixel squares was averaged, background subtracted, and normalized to the intensity at 0 min (Yang et al., 2007).

2.5. Statistics

All data are presented as means ± sem for $N = 10$ slices per condition. Where not present, error bars did not exceed size of the plotted symbol. Statistical analyses ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$) were performed using Student's two-tailed unpaired *t*-tests or two-way ANOVAs followed by either Sidak or Tukey post test multiple comparisons (GraphPad Prism v6.01).

3. Results

3.1. SEL decreased synaptic responses in a time-, frequency- and concentration-dependent manner

Field excitatory postsynaptic potentials (fEPSPs) recorded from CA1 synapses in response to a train of 20 pulses delivered at 40 Hz initially potentiated and subsequently decreased in amplitude over the course of the train (Fig. 1A). This short term depression (STD) was only slightly affected by 30 min exposure to 30 μM SEL (Fig. 1B–E). However, a 3 h incubation in 30 μM SEL greatly enhanced the STD (Fig. 1). Plots of normalized fEPSP amplitudes